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# Differential effect of Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) on leukocyte infiltration during contact hypersensitivity responses

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BACKGROUND: 2’-4’ Dinitrofluorobenzene (DNFB) induced contact hypersensitivity is an established model of contact sensitivity and leukocyte migration. Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) deficient mice were used to examine the role of PECAM-1 in the migration capacity of several different leukocyte populations after primary and secondary application. RESULTS: γδ T lymphocytes, granulocytes, and Natural Killer cells were most affected by PECAM-1 deficiency at the primary site of application. γδ T lymphocytes, granulocytes, DX5+ Natural Killer cells, and, interestingly, effector CD4+ T lymphocytes were most affected by the loss of PECAM-1 at the secondary site of application. CONCLUSIONS: PECAM-1 is used by many leukocyte populations for migration, but there are clearly differential effects on the usage by each subset. Further, the overall kinetics of each population varied between primary and secondary application, with large relative increases in γδ T lymphocytes during the secondary response.

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1 **Differential effect of Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) on**
2 **leukocyte infiltration during contact hypersensitivity responses.**
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29

30 Abbreviations:

31 DNFB 2'-4' Dinitrofluorobenzene

32 PECAM-1 Platelet Endothelial Cell Adhesion Molecule-1

33 CD Cluster of Differentiation

34 NK Natural Killer Lymphocyte

35

**Abstract**

BACKGROUND: 2'-4' Dinitrofluorobenzene (DNFB) induced contact hypersensitivity is an established model of contact sensitivity and leukocyte migration. Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) deficient mice were used to examine the role of PECAM-1 in the migration capacity of several different leukocyte populations after primary and secondary application.

RESULTS: γδ T lymphocytes, granulocytes, and Natural Killer cells were most affected by PECAM-1 deficiency at the primary site of application. γδ T lymphocytes, granulocytes, DX5+ Natural Killer cells, and, interestingly, effector CD4+ T lymphocytes were most affected by the loss of PECAM-1 at the secondary site of application.

CONCLUSIONS: PECAM-1 is used by many leukocyte populations for migration, but there are clearly differential effects on the usage by each subset. Further, the overall kinetics of each population varied between primary and secondary application, with large relative increases in γδ T lymphocytes during the secondary response.

52

53 **Introduction**

54 2'-4' Dinitrofluorobenzene (DNFB) induced contact hypersensitivity is an
55 established model of inflammation. Haptenylation of proteins by DNFB induces a type
56 IV delayed hypersensitivity [1]. One of the most important recent advances in the field
57 of Natural Killer (NK) lymphocyte immunology was the discovery that not only do NK
58 cells contribute to this response, but also that they have a memory-recall response
59 which resides in a liver CXCR6+ population [2, 3].

60

61 Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1, CD31) is expressed on
62 platelets, endothelial cells and all leukocytes, while it is shed on activated B and T
63 lymphocytes [4-6]. PECAM-1 is expressed at endothelial cell junctions and PECAM-1
64 homophilic interactions are important as leukocytes undergo extravasation into
65 inflamed tissues [7].

66

67 The role of PECAM-1 had not previously been studied in the context of DNFB-
68 induced contact hypersensitivity nor on γδ T and NK lymphocytes. In this study, we
69 tested both the primary and secondary responses of γδ T and NK lymphocytes in wild
70 type and PECAM-1 deficient mice. There are relatively few papers studying the effects
71 of DNFB on γδ T cells [8-10] but this population plays an important role in skin contact
72 hypersensitivity reactions.

73

74 **Materials & Methods**

*Ethics Statement:* Colorado State University Institutional Animal Care and Compliance committee approved all protocols related to this project (Approval ID 05-153).

*Animals*

Age (2-4 months) and gender matched male and female wild type and PECAM-1 deficient mice in the FVB/n strain were used for all experiments. Due to the spontaneous pulmonary fibrosis in the PECAM-1 deficient mice in this strain [11], any animals showing disease were excluded after screening by blood oxygen saturation [12]. For all experiments, 5-6 wild type or PECAM-1 deficient mice were used in each group. Each experiment (primary or secondary exposure) was repeated 3 times.

*Primary DNFB exposure*

25ul of 0.5% DNFB in olive oil was applied on ~1cm$^2$ unshaved flank skin by directly pipetting onto the surface. We used unshaven skin because sometimes shaving caused skin irritation. Skin was harvested 48 hour later for histologic and flow cytometric analysis. For flow cytometry, skin was trimmed to 3cm X 5 cm sections before digestion and cell counts.

*Secondary DNFB exposure*

Five days after primary exposure, 10ul of 0.15% DNFB in olive oil was applied to one ear; the opposite ear was also treated with olive oil to account for grooming of the skin. 48 hours later ears were harvested and fixed in 10% buffered formalin for histology or digested in collagenase for flow cytometry.

98 *Flow cytometry*

99 Ears or skin were incubated in RPMI 1640 (Life Technologies, Gaithersburg, MD)
100 containing collagenase IV (0.7 mg/ml) for 30 minutes, then RPMI containing 10% fetal
101 bovine serum was added to stop digestion. The entire mixture was filtered through
102 40um filters to collect cells by centrifugation. Cold phosphate buffered saline was used
103 for all further staining steps. Cells were pre-blocked in 1% normal rat serum and Fc-
104 blocking antibodies before staining with the following antibodies or isotype-matched
105 color controls.

106

107 All antibodies (Table 1) and isotype color controls were purchased from eBioscience
108 (San Diego, CA) except for CD206 (Biolegend, San Diego, CA). Clones indicated in
109 parentheses. Flow cytometry was performed on a Dako Cyan (Carpinteria, CA) and
110 analyzed using FlowJo (Ashland, OR) and JMP statistical (SAS, Cary, NC) software.

111

112 Gating scheme: Live/dead exclusion was not used because some cells might be
113 missed (dying neutrophils) and few dead cells were found outside of the typical debris
114 or doublet gates. CD3+ CD4+ or CD3+ CD8+ T cells were analyzed for CD44 and
115 CD62L markers of activation status. CD19+ CD3- B cells were analyzed for CD5 and
116 IgD. NK cells were defined as CD3-, γδ TCR -, NKG2D+, DX5/CD49b+. CD3+ γδ
117 TCR+ T cells were also defined by CD8 high or low. Neutrophils were defined by GR1+
118 CD11b+ CD11c$^{low/neg,}$ CD115-. Dendritic cells were defined as CD11c+ CD11b$^{low}$.
119 Monocytes and macrophages were defined as CD11b+ CD11c+ CD115+ [13] and

120 personal communication with Dr. Gwendalyn Randolph (Washington University, St.
121 Louis, MO).
122
123 *Statistics*
124 Samples from primary and secondary exposures to DNFB were done a minimum of
125 three times with 4-6 mice in each group (PECAM-1 deficient mice and wild type mice)
126 after testing for power. JMP statistical software (SAS, Cary, NC) was used to analyze
127 differences between groups using Tukey-Kramer Honestly Significant Different or One
128 Way ANOVA tests.
129
130
131 **Results**
132 The immune response to primary DNFB exposure in PECAM-1 deficient mice has
133 not previously been reported. Upon dissection of the flank skin at the site of a single
134 DNFB exposure, we found that the blood vessels were more dilated around the major
135 vessels in PECAM-1 deficient mice than in wild type mice (Figure 1).
136 Somewhat paradoxically to the gross appearance, the flank skin layer had a larger
137 inflammatory infiltrate of cells in wild type mice, primarily mononuclear cells with
138 relatively few neutrophils (Figure 2A, 2C) compared to PECAM-1 deficient mice (Figure
139 2B, 2D). After collagenase digestion of flank skin tissue and collection of cells, we found
140 significantly fewer total cells in PECAM-1 deficient mouse skin (Figure 3A) despite using
141 equal sized (3cm X 5cm) sections from both mice. Of the cells that were positive for the
142 leukocyte panels we used, most leukocytes in the dermis were monocyte/macrophage

143 phenotype, with differential effects in the two major populations CD11b$^{low}$/CD11c$^{high}$
144 "resident" macrophages not as affected as CD11b$^{high}$/CD11c$^{low}$ monocyte/small
145 inflammatory macrophages (Figure 3b). γδ TCR+ lymphocytes and CD11b$^{high}$/CD11c$^{high}$
146 dendritic cells were significantly lower in PECAM-1 deficient mice. Using two markers,
147 NKG2D and DX5, for Natural Killer cells, we found they were significantly lower in
148 PECAM-1 deficient mice as well. CD8$^{bright}$ CD3+ cells were found at low levels and not
149 significantly lowered by PECAM deficiency although numbers were lower overall.
150 Upon the secondary challenge five days later in the ear, ear redness and swelling
151 were not significantly different between wild type and PECAM-1 deficient mice (Fig 4).
152 However, the observed number of inflammatory cells was significantly lower in PECAM-
153 1 deficient mice (Figure 5). γδ TCR+ cells (Figure 6A), neutrophils (Figure 6B), dendritic
154 cells, DX5+/NKG2D+ cells, and classical CD4+ and CD8+ cells (Figure 6C) were much
155 lower overall. "Resident" macrophages (Figure 6D) appeared to be mostly unchanged
156 and were by far the largest population. PECAM-1 deficiency significantly reduced the
157 influx of some populations (effector CD4+ CD62L-/CD44+ T cells, NK cells, neutrophils,
158 and γδ TCR+ cells) but not others (effector CD8+ CD62L-/CD44+ T cells, and dendritic
159 cells). It was most striking that the cells most mobilized by the secondary inflammatory
160 response (neutrophils and γδ TCR+ cells) were also most affected by PECAM-1
161 deficiency.
162

163 **Discussion**

164 This study shows two important results. First, the high participation of
165 DX5+/NKG2D+ phenotype cells in the primary immune response to DNFB does not

166 necessarily correspond to a similarly proportioned response at a secondary challenge.
167 Likewise, γδ TCR+ cells were relatively minimal (~1% of total) in the primary challenge
168 site but became quite vigorously involved in the secondary response, outnumbering any
169 other single population (other than resident macrophages) by almost 5-fold.

170 Second, PECAM-1 deficiency manifested itself in differential effects on each
171 population. Most were affected by the deficiency, but it was surprising that CD8+ T
172 effectors were able to overcome the lack of PECAM-1 whereas CD4+ T cell infiltration
173 was clearly altered. We did not look for CD4+ CD3+ T cells at the primary exposure site,
174 as we did not expect to find many in the skin as we thought the primary response would
175 be largely driven at this stage by macrophages and NK cells, and that CD4 responses
176 would be activated later by antigen-presenting cells. Further, it would be fascinating to
177 examine the effects on CD4+ T cells at both the primary skin flank exposure site and the
178 ear as CD4 cells do play a significant helper role in the response [14]. Clearly CD4 help
179 at both sites may potentially drive some activation and differential effects on CD8 and
180 γδ TCR+ cells at both sites. DX5+/NKG2D+ cells, being innate, may in turn be waning
181 in their responses as the adaptive immune response takes over the inflammatory
182 process.

183 Another leukocyte population we did not examine is the Innate Lymphoid cells
184 (ILCs), which were largely unknown at the time these studies were performed [15]. NK
185 cells are also part of the Innate Lymphoid cell family, and it makes logical sense to
186 explore the role of ILC1, ILC2, and ILC3 subsets in this response. Additionally, we did
187 not look at cell counts for fibroblasts, epithelial cells, endothelial cells, which may
188 comprise a large number of the total cells collected (Figure 3A). A more holistic

189 approach to all of the cells involved could be very informative as it was also somewhat
190 surprising to see the differential effects on the vasculature dilation (Figure 1) in PECAM-
191 1 deficient strain at the site of primary exposure. As PECAM-1 is vital to endothelial cell
192 permeability [16, 17], this study shows quite dramatically the role of this molecule to the
193 earliest stages of the inflammatory response in this model as well.

194 There is considerable evidence that γδ TCR+ cells play a significant role in skin
195 allergies and hapten-induced contact sensitivity [10]. Many of them also expressed
196 NKG2D, and NKG2D played a role in the activation cycle [18]. More recently, the
197 contribution of Vγ4 T cells in the response to DNFB and subsequent neutrophil
198 infiltration was elegantly mapped out by Jiang et al. [19] Our work largely complements
199 those studies, though it is important to note that their use of the term "primary exposure"
200 still is measured after a second exposure to DNFB.

201 Our studies do pose many questions to answer in possible future studies. In
202 particular, we would like to pursue a longer lag time between primary and secondary
203 challenges. For consistency, we used the classical assay used by many other groups
204 with a total of 7 days between primary challenge and harvest after secondary challenge
205 [1, 2, 20]. Perhaps the response kinetics would be altered between each population. It
206 has previously been shown that memory recall response by NK cells in mice can last as
207 long as four months [3].

208 We would also would have preferred to use NK1.1 as a marker for confirmation of
209 the NK phenotype. Unfortunately, we did not use NK1.1 on FVB/n mice as we only
210 retrospectively discovered that FVB/n mice do indeed express NK1.1 [21]. Finally, it
211 would be very important to identify which γδ TCR population(s) are mobilized, as there

212 is tissue specific localization of the γδ TCR subsets. The Vγ3 subset is commonly found
213 in skin [22], however it would be interesting to assess whether any blood Vγ1, Vγ2 or
214 epidermal Vγ5 [23] would be mobilized or differentially affected by PECAM-1 deficiency,
215 similar to the different effects on CD4+ and CD8+ T cells.
216 Unfortunately, this strain of mice was lost to future studies at this time due to poor
217 breeding and limited lifespan due to spontaneous pulmonary fibrosis [12]. There is a
218 PECAM-1 deficient mouse in the C57BL/6 background. The C57BL/6 surprisingly has a
219 near-normal response in many inflammatory models compared to wild type mice [24],
220 whereas the FVB/n strain exhibited the phenotype we expected with markedly reduced
221 inflammatory responses. This difference mapped to a locus on chromosome 2 [25]. The
222 C57BL/6 strain is also resistant to the pulmonary fibrotic disease [11], and this appears
223 to be related to vascular function [17]. It would be interesting to again compare and
224 contrast these strains and the effects on their populations in future studies. However,
225 we believe any subsequent studies would be best conducted with cell-targeted gene
226 disruption of various subsets like monocytes, natural killer lymphocytes, or γδ TCR+ to
227 also further determine the roles of these subsets in the response to DNFB.
228
229 Competing interests: The authors state they have no competing or conflicts of financial
230 or non-financial interest.
231
232 Authors' contributions:
233 M.E., W.G.S., R.U., J. M. G., M.L., and A.R.S. did the animal care and exposure
234 studies, including tissue harvest, flow and histological preparation, and data analysis.

Mice, funding as principle investigator, and data analysis was supplied by W.A.M.. All authors read and approved the final manuscript.

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246 **Figure Legends**

247 Figure 1. Gross anatomy of the skin at the flank site of primary DNFB application 28
248 hours after application. The dilation of vasculature is much more prominent in the
249 PECAM-1 deficient mice. Lower panels show higher magnification.

250

251 Figure 2. Representative sections of inflammation in primary flank skin exposure to
252 DNFB from wild type (A, 2X magnification; C, 20X magnification) and PECAM-1
253 deficient (B, 2X magnification; D, 20X magnification) mice.

254

255 Figure 3.Total (A) and leukocyte subset (B) cell counts from wild type and PECAM-1
256 deficient flank skin at the treatment sites, 48 hours after DNFB application. * $p < 0.05$, **
257 $p < 0.01$ Tukey Kramer HSD test. ns = not significant difference.

258

259 Figure 4. Right ear swelling by skin caliper measurement upon secondary exposure to
260 DNFB in wild-type and PECAM-1 deficient mice. Left ears were measured as controls.

261

262 Figure 5. Representative ear sections after DNFB treatment. A-B: DNFB treated wild
263 type ears. C-E: DNFB treated PECAM -/- ears. F: Untreated wild type control, PECAM-/-
264 were equivalent in examination. 20X magnification.

265

266 Figure 6. Cell counts from treated (right) and untreated (left) ears. Larger populations
267 (A, B, and D) are separated out for clarity. A. γδ TCR+ cell counts. B. Granulocytes. C.
268 Other lymphocyte populations. D. Resident macrophages, by far the largest population,

were largely unaffected by DNFB treatment. * $p < 0.05$, Tukey Kramer HSD test. ns = not significant difference.

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# Table 1 (on next page)

Flow Cytometry Reagents Used

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| Color | CD4+ T | CD8+ T | CD19+ B | NK/γδ | Myeloid Cells |
|---|---|---|---|---|---|
| **APC CY7** | CD3 (145-2C11) | CD3 | CD3 (-) | CD3 | |
| **PE CY5** | | | | | |
| **Pacific Blue** | CD4 (L3-T4) | CD8 (53-6.7) | CD19 (6D5) | CD8 | CD11c (N418) |
| **FITC** | CD44 (IM7) | CD44 | CD5 (53-7.3) | pan-γδ T cell (GL3) | CD11b (M1/70) |
| **APC** | CD62L (MEL-14) | CD62L | CD62L | | GR1 (RB6-8C5) |
| **PE Cy7** | | | | NKG2D (CX5) | CD206 (C068C2) |
| **PE** | | | IgD (11-26c) | DX5 (CD49b) | CD115 (AFS98) |

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## Figure 1(on next page)

Gross anatomy of the skin at the flank site of primary DNFB application 28 hours after application.

The dilation of vasculature is much more prominent in the PECAM-1 deficient mice. Lower panels show higher magnification.

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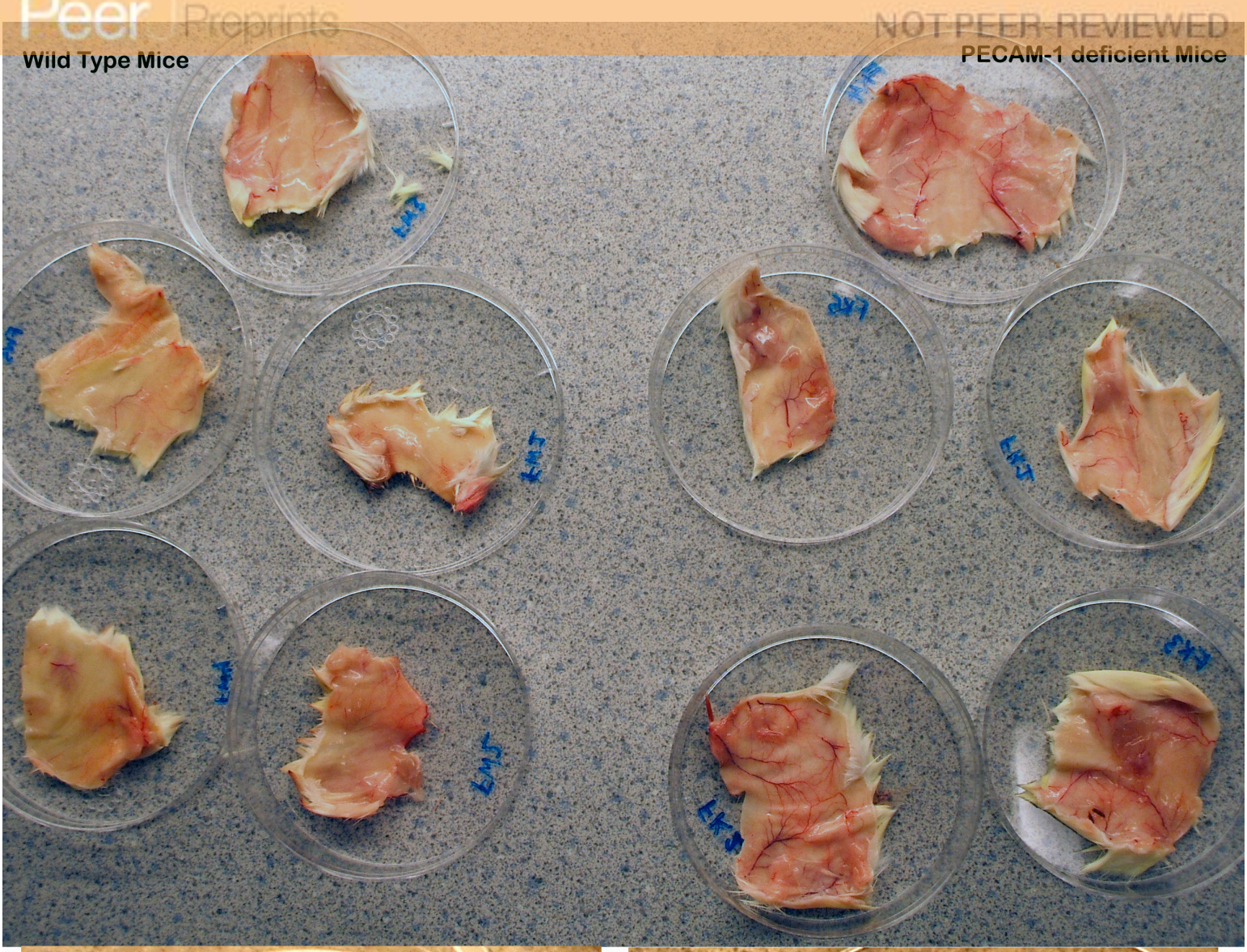

## Figure 2(on next page)

Inflammation in primary flank skin exposure to DNFB

Representative sections of inflammation in primary flank skin exposure to DNFB from wild type (A, 2X magnification; C, 20X magnification) and PECAM-1 deficient (B, 2X magnification; D, 20X magnification) mice.

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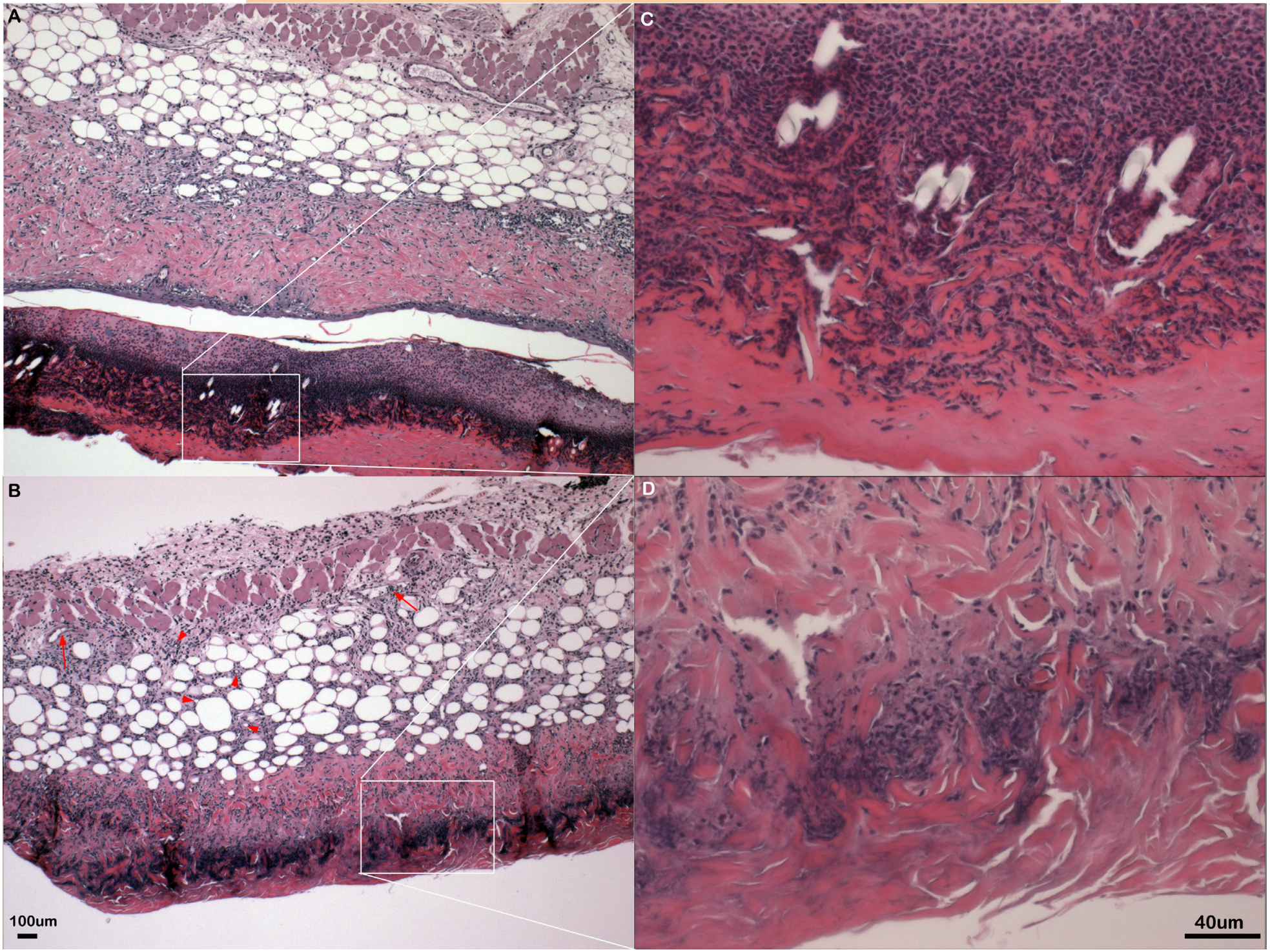


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## Figure 3(on next page)

Leukocyte counts at primary DNFB exposure flank skin

Total (A) and leukocyte subset (B) cell counts from wild type and PECAM-1 deficient flank skin at the treatment sites, 48 hours after DNFB application. * $p < 0.05$, Tukey Kramer HSD test. ns = not significant difference.

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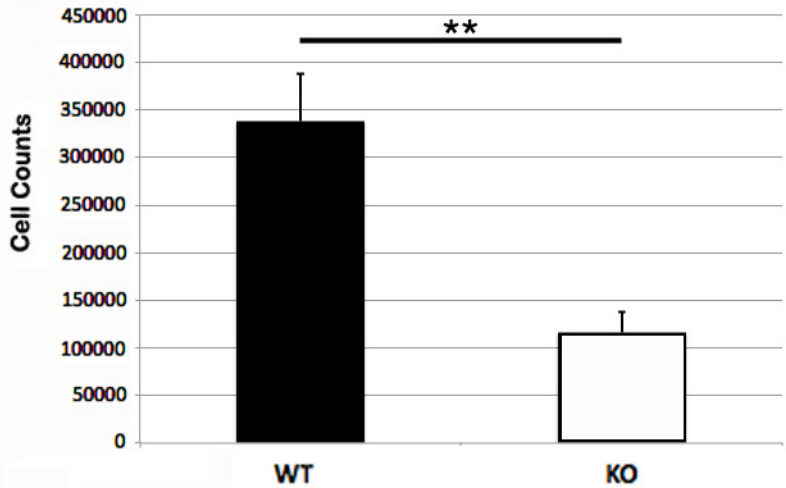
A

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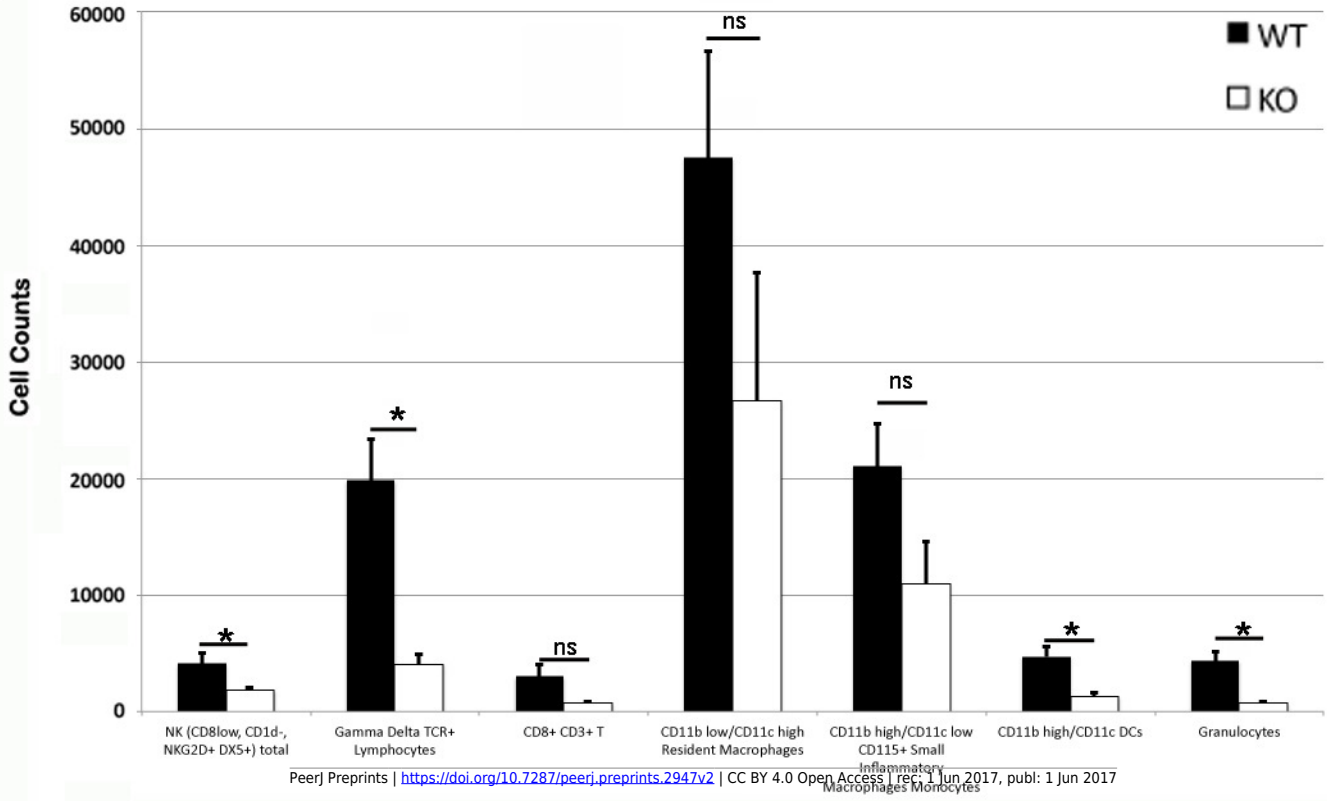


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## Figure 4(on next page)

Ear swelling at secondary challenge

Right ear swelling by skin caliper measurement upon secondary exposure to DNFB in wild-type and PECAM-1 KO mice. Left ears were measured as controls for each animal.

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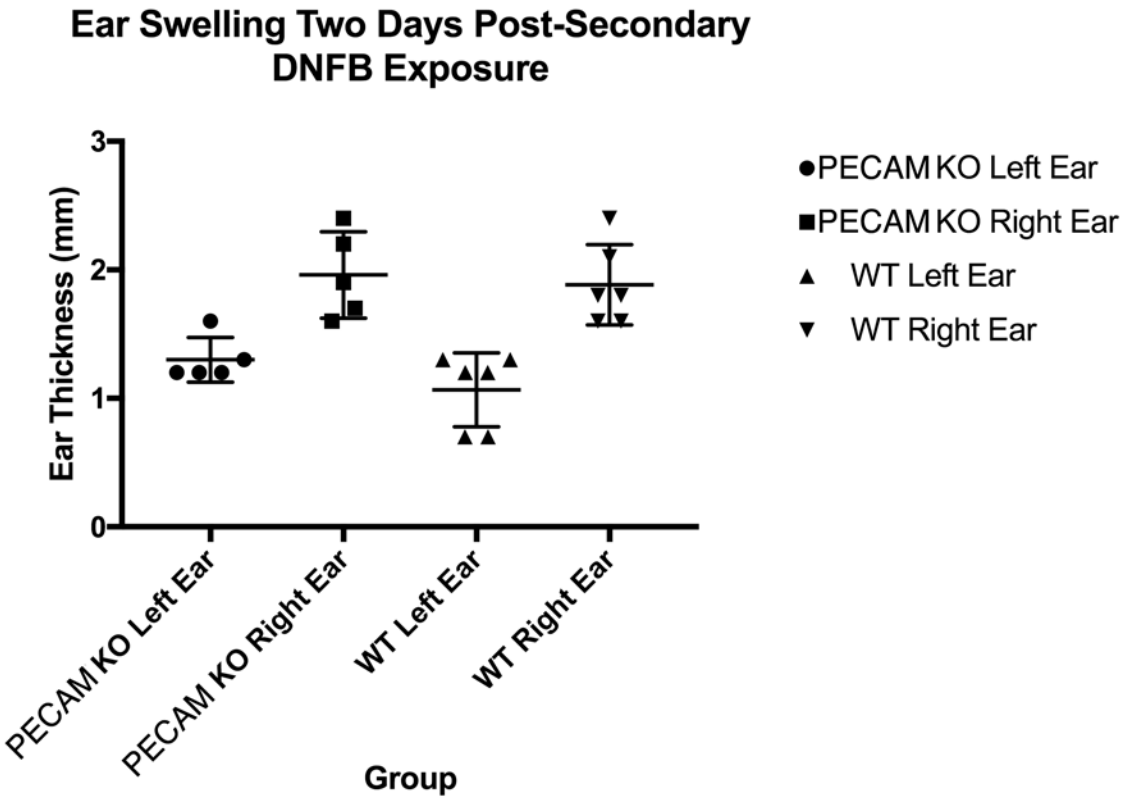


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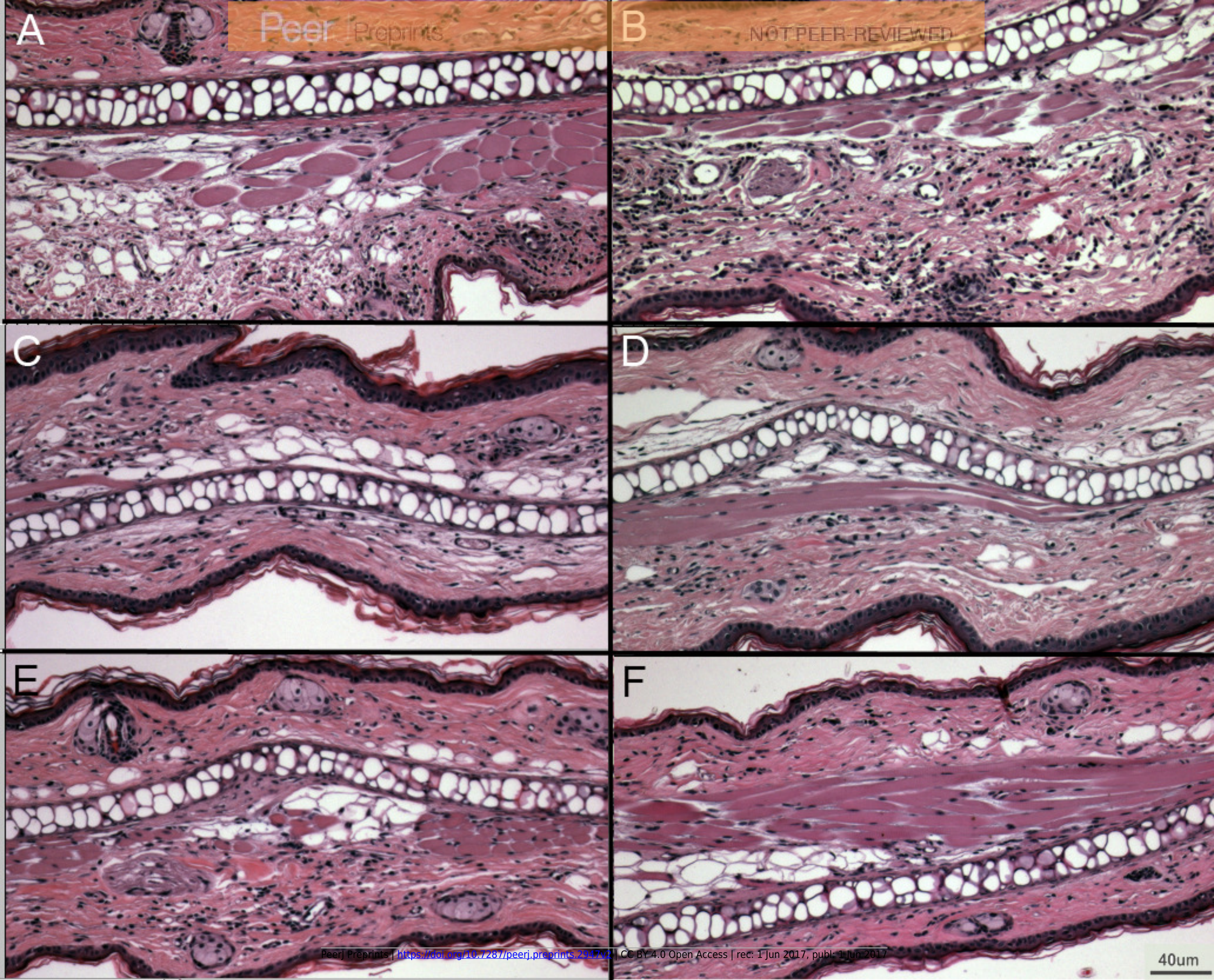
## Figure 5(on next page)

Representative ear sections after DNFB treatment.

A-B: DNFB treated wild type ears. C-E: DNFB treated PECAM KO ears. F: Untreated wild type controls and PECAM KO were equivalent in examination. 20X magnification.

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## Figure 6(on next page)

Cell counts from treated (right) and untreated (left) ears.

Larger populations (A, B, and D) are separated out for clarity. A. γδ TCR+ cell counts. B. Granulocytes. C. Other lymphocyte populations. D. Resident macrophages, by far the largest population, were largely unaffected by DNFB treatment. * $p < 0.05$, Tukey Kramer HSD test. ns = not significant difference.

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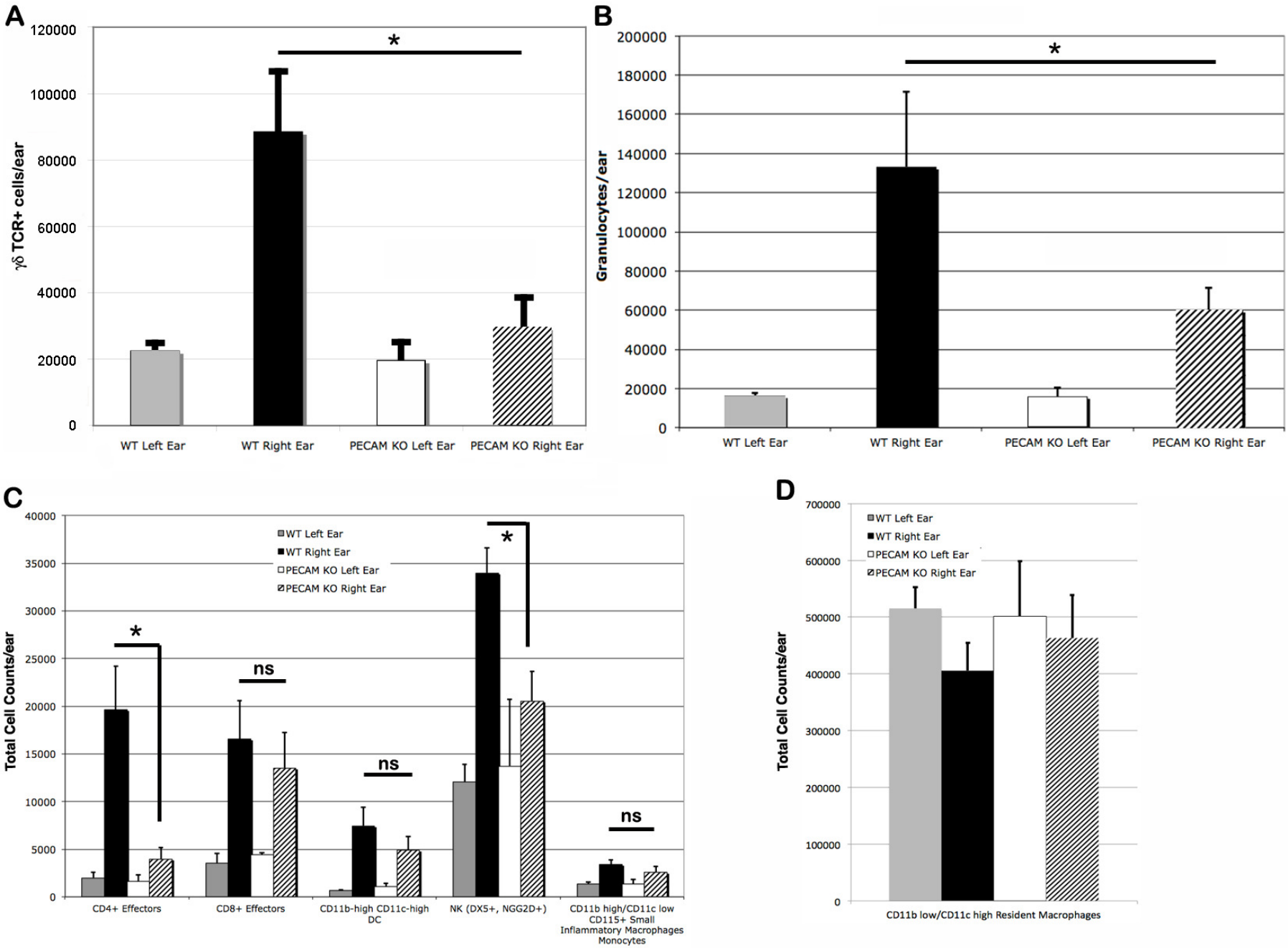


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